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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Eric A. Schon

Serial No.: 08/409,644 Examiner: J. Fredman

Filed: March 24, 1995 Group Art Unit: 1807

For: A METHOD TO DETECT MUTATIONS IN A NUCLEIC ACID USING A HYBRIDIZATION-LIGATION PROCEDURE

1185 Avenue of the Americas
New York, New York 10036
February 29, 1996

Assistant Commissioner of Patents
Washington, D.C. 20231

DECLARATION OF ERIC A. SCHON PH.D. UNDER 37 C.F.R. § 1.131

Sir:

I, Eric A. Schon, hereby declare as follows:

1. I am the sole inventor named on the above-identified patent application.

2. The invention claimed in the above-identified application was conceived solely by me, and either directly or through persons acting under my direction and supervision, actually reduced to practice in the United States prior to September 30, 1994.

3. As evidence of the fact that the invention claimed was actually reduced to practice in the United States prior to September 30, 1994, I have annexed hereto as Exhibits 1-4 copies of pages from the laboratory notebook of my technician, Mr. Jeffery S. Rogers. These copies are true and accurate copies except that the dates have been

redacted. All of the redacted dates are prior to September 30, 1994.

4. The claimed invention is a method for detecting the presence or absence of a predefined mutation in a nucleic acid. This method was specifically used to detect the presence of a known mutation in the MELAS-3243 nucleic acid. The claimed invention involves contacting the nucleic acid molecule with a linear probe comprising two covalently linked nucleic acid segments under conditions such that the unlinked end of each segment of the probe is capable of hybridizing with the nucleic acid molecule. As shown in Exhibit 1, a probe designated the LiCat-Melas.1 probe was synthesized and radiolabeled. As shown in Exhibit 2, the mutant MELAS-3243 nucleic acid was contacted with the LiCat-Melas.1 probe, under suitable hybridization conditions to form a hybridization product.

5. The claimed invention further involves contacting the hybridized product from paragraph 4 above, with a ligase under conditions such that the unlinked ends of the segments ligate together if the nucleic acid molecule contains the mutation. As shown in Exhibit 3, the hybridization product was contacted with T4 DNA ligase under suitable ligation conditions.

6. The claimed invention further involves determining whether the unlinked ends of the segments have ligated together so as to thereby detect the presence or absence of the mutation in the nucleic acid molecule. As shown in Exhibit 4, the hybridization product was then electrophoresed through a 0.8% agarose gel, dried under vacuum and then subjected to autoradiography in order to

determine whether ligation has occurred. Ligation had occurred. In this way the presence of the predefined mutation in the nucleic acid molecule was detected.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of United States Code and that false statements may jeopardize the validity of the application or any patent issuing thereon.

Eric A. Schon, Ph.D.

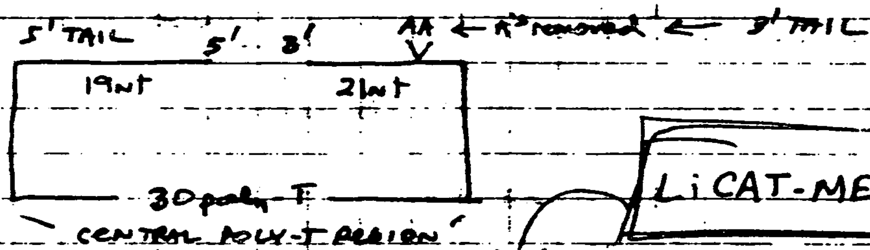
Feb. 29, 1996

Eric A. Schon, Ph.D. Date

– Genosys Corp can generate 80-mer oligos
(3' nt of this oligo will be very pure)

[Biotinylate dTTP central nt's?]

[TEST PRIMER 70-mer]: shorten 3' end by 2 AA's (underlined).
central poly T region = 30nt.



LiCAT-MELAS.1

SEQUENCE:

5' END TAIL — CENTRAL "POLY-T" REGION

5'– CTG/CCA/TCT/TAA/CAA/ACC/CTT/TTT/TTT/TTT/TTT/TTT/TTT/

3' END TAIL

TTT/TTT/TTT/TGT/TTT/ATG/CGA/TTA/CCG/GGC/C –3'

These 2 T's replace AA's which were originally part of 3' TAIL. — 3243

* ordered from DNA Synthesis Facility

→ There may be a problem with synthesizing a primer with this many polynucleotides (TTP). Sometimes chain will fold back on itself and fail to extend during synthesis. → Genosys can do this with no problem

TEMPLATES

pCR16.3/16.4 would be good (plasmid is 5.3kb) control.

Patient DNA's: Stow | Ferrow | Nardi | Rowsden — MELAS 3243+ Available?

obtained from Yasu: WS176: MELAS+ Cybrid (homoplasmic)
WS239 wt Cybrid (homoplasmic)

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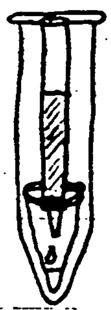
Exhibit 1

[redacted]

II. CLEAN LABLED PRIMER: (Maniatis technique Book III pg. E37-E38)

1. Add 80λ TE to reaction tube (final volume = 100λ)
2. Spin out TE of prepared G-25 column: (G-25 sephadex - Medium)
   a. 1ml tuberculin syringe → plug bottom w/ glasswool
   b. Add G-25 slurry to syringe (make sure G-25 doesn't flow out)
   c). Spin syringe on table top centrifuge (speed #3) 2'
   (Place syringe in 15ml Corning tube for support)
   d). Repeat until packed column (looks slightly dessicated is 4/1.5" high)
   e). Add fresh TE to top of column + spin through
   Repeat this step 3-4 times to wash column.
   f) Plug luer tip with white cap. Add fresh TE to top of column. Parafilm top. Store +4°C in upright stance.
3. Add 100λ of Primer RxN mix to top + center of stacked G-25 bed.
4. Place syringe in fresh microfuge tube



5. Spin column on #3 x 4'
6. Check μtube for collected 100λ → this is labled product
7. Check colum for collected radioactivity + check product.

MEASURE SPECIFIC ACTIVITY:

1. Estimate volume. Add 1λ of product to 5mls Scintillation Soln
2. Measure on Spec: = 189745 CPM/λ × 100λ = 1.9×10^7 CPM

F#	S#	TIME	CPMA/K	%DEV	CPMB/K	%DEV	CPMC/K	%DEV	SIE	SIS F
6	→1	1.00	189745.	.46	158625.	.50	.00	.00	.000	690.02
6	Blank 2	1.00	20.00	44.7	7.00	75.5	.00	.00	.000	19.716
			94882.5		79315.0		.00		.000	354.85
6	^{14}C 3 STD	1.00	112084.	.60	54382.0	.86	.00	.00	.000	171.15
6	STD. 4	1.00	27.00	38.4	16.00	50.0	.00	.00	.000	25.563
			56055.5		27199.0		.00		.000	98.357

$\frac{1.9 \times 10^7 \text{ CPM}}{9.2\,ug \ (140 pmol)} = 2 \times 10^6$ CPM/ug

4.8×10^5 CPM/pmol

Apologies LIGHT

40pmol / 100λ = 0.4 pmol/λ (10pmol = 25λ)

↑ LABELED PRIMER CONC

HYBRIDIZATION ((10:1) Primer / Template Ratio) PCR 16.3/16.4 ONLY

IN tube:
- 25λ LICAT-MELTS.1 Primer (10pmol)
- 1λ pCR16.3/16.4 (≈1.5ug = ≈1pmol)
- 10λ 10X ANNealing Buffer (See Below)
- 64λ DDW

100λ TOTAL

↓

Boil x 5' (Denature Plasmid DNA)

↓

55° 2 HRS

(Eric thinks primer/template annealing will be rapid. → suggested shorter time to prevent plasmid re-annealing.)

	1pmol = X ug
LICAT-MELTS	1pmol = 0.23ug
pCR16.3/16.4	1pmol = 1.8ug

10 X ANNEALING BUFFER
- 500mM NaCl
- 100mM $MgCl_2$
- 100mM TRIS-Cl pH 7.4

1. Remove Hybridization tubes from 55°C bath → Place on ice.
2. Add 0.2 x vol of 10M NH_4OAc (20λ) → mix
3. Add 240λ ice-cold 100% Ethanol → mix. → Store on ice 15-30'. (The hybridized DNA's will co-precipitate as a unit. Along with un-hybridized DNA's.)
4. Centrifuge 15' @ 13,000 RPM.
5. Remove S°. Wash gently 2x w/ RT 70% Ethanol (crude pellet for radioactivity)
6. Resuspend pellet IN. 10λ DDW. → remove 5λ of this mixture and store -20°C for step control

↓

~~Assays~~ L CAT

cont.

oops - mistake made s/b 10:1 → (1:1 PRIMER/TEMP RATIO)

To lyophilized pellet add:
- 2λ LCAT-MELAS.1 Primer (0.8 pmol) (1/29 washed Sample)
- 2λ 10X Hyb. Buffer
- 16λ DDW

↓

65°C x 2' → Slow Cool <35°C x ~45'

↓

Spin Briefly → Save 5λ (+Hyb −LiG)

↓

LIGATIONS

+CONTROL
- 5λ λHindIII DNA (0.5ug)
- 1λ T4 DNA Ligase
- 2λ 10X Ligase Buffer
- 12λ DDW
- 20λ

- 10λ Template/Primer Anneal Mix
- 2λ 10X Ligation Buffer (Ent)
- 1λ T4 DNA Ligase
- 7λ DDW
- 20λ

↓

12-15°C Bath x ~~[illegible]~~ O.N. (2/1 2:21 PM → 2/2 10:30 AM ~20 HRS.)

→ − Inactivate Ligase → 65°C x 15'

− Remove 10λ of Ligation mix to separate tubes (+LiG +HYB)

Of the remaining 10λ: Add
- 10λ mix
- 0.8λ 5mM EDTA
- 4λ 1N NaOH
- 5.2λ DDW

↓

~~RT~~ x ~~10'~~ ~30' on ice

↓

+2λ 10X Dye

Load denatured DNA directly onto gel do not delay.

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Exhibit 3

PREPARE 0.8% TAE AGAROSE GEL:

LANE/LOAD

1 –	λ HIND III Marker (0.5 ug of λ HIND III used in ligation + CONT.)		
2 –	pCR16.3	1λ Plasmid	
3 –	pCR16.4	"	
4 –	pCR 16.3	+HYB/-LIG	(No Denature) (Use half)
5 –	pCR 16.4	+HYB/-LIG	(No Denature) (Use half)
6 –	pCR16.3	+HYB/+LIG	(")
7 –	pCR16.4	+HYB/+LIG	("
8 –	pCR16.3	" "	+ Alkaline Denatured
9 –	pCR16.4	" "	" "
10 –	λ LIGATION + CONTROL		

~~2ND ROW~~

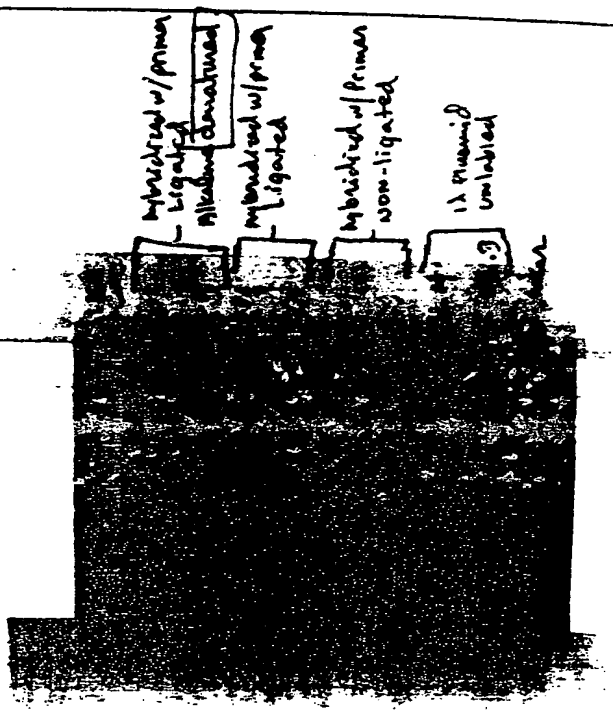
~~1 –~~	~~λ HIND III Marker~~		
2 –	pCR16.3		
3 –	pCR16.4		
4 –	pCR16.3	+HYB/-LIG	Denatured (Alkaline)
5 –	pCR16.4	+HYB/-LIG	" "

Gel dried on vacuum 2 HRS

↓

Expose + Screen

–80° C



WITNESSED: [illegible]

RESULTS: IT WORKS! The catenane of pCR16.3 (mels+) + LICAT mels.) Primer appears to be resistent to denaturation + loss of signal. Even the 65°C Ligase killing step (post-ligation) makes the wt template lose the primer, but not the mels+ template

NEXT STEP:

1) Repeat experiment using Cybrid total DNA's (Liquid RxN ⇒ hyb/lig etc.) mels+ mels−

2) Spot solid matrix (e.g. Zeta-probe GT membrane) with primer + hyb/ligate/denature with pCR-1000 labeled probe.. (~~same matrix technical problems~~)

? 1 membrane material interfere with ligation reaction??

? 2 Do reations on punched holes of membrane IN 96-well TC dish?

? 3 STRIPS of membrane?

WS176 = mels+ } both
WS239 = wt } homoplasmic

LICAT.MELAS.1 + CYBRID TOTAL DNA

I. SPECTROPHOTOMETER MEASURE (@ 37°C)

	IN TEPH8	A^{260}	A^{280}	
1. WS176 (MELAS+)	1:1000 DiLN.	.044	0	440.D.
2. WS239 (wt)	1:1000 DiLN.	.010	0	100.D.

say roughly 10% of yield is from mtDNA

mels+ = 2200 ug/ml = 2.2 ug/λ TOTAL DNA → 0.2 ug/λ

wt = 500 ug/ml = 0.5 ug/λ " " → 0.05 ug/λ

mtDNA yield

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